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ANTENNULAR CHEMOSENSITIVITY IN THE SPINY LOBSTER, *PANULIRUS ARGUS:* COMPARATIVE TESTS OF HIGH AND LOW MOLECULAR WEIGHT STIMULANTS

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B. W. ACHE, Z. M. FUZESSERY, AND W. E. S. CARR

Department of Biological Sciences, Florida Atlantic University, Boca Raton, Florida 33431, and Whitney Marine Laboratory, University of Florida, Rt. 1, Box 121, St. Augustine, Florida 32084

The role of proteins and other macromolecules as feeding stimuli for marine invertebrates is not clearly defined. Macromolecules can be effectively deployed in the aquatic environment (Wilson, 1970), but the extent to which marine organisms utilize such molecules is the subject of some controversy among students of chemical communication (Heeb, 1973). Behavioral studies implicating macromolecules acting as feeding stimulants in polychaetes (Magnum and Cox, 1971), natantians (Carr and Gurin, 1975), asteroids (Heeb, 1973), and gastropods (Gurin and Carr, 1971; Carr, Hall, and Gurin, 1974) counter the trend of thinking established by earlier studies that low molecular weight, readily diffusable organic molecules are the predominate feeding stimuli for marine invertebrates (for review see Lenhoff and Lindstedt, 1974). Further investigation of the stimulatory role of macromolecules is in order.

Electrophysiological analysis of chemoreceptor sensitivity to macromolecular stimuli provides a novel approach to evaluating the potential of macromolecules as feeding stimuli. Case (1964) demonstrated that polyglutamic acid and two proteins were not stimulatory to dactyl chemoreceptors of the brachyuran, *Cancer antennarius*. From Wilson's (1970) consideration, proteins serving as contact chemical stimuli raises fewer conceptual problems than proteins serving as distance stimuli where increased emission rates, lowered receptor thresholds, and/or current-facilitated dispersal are necessary to offset the low diffusion coefficients of proteins in water. An analysis of distance chemoreceptors would therefore be particularly appropriate to understanding the potential stimulatory capacity of macromolecules.

Behavioral and electrophysiological data implicate the antennules of decapod crustaceans as distance chemoreceptors (Hazlett, 1971). The spiny lobster, *Panulirus argus,* with its elongated antennular filaments is well suited for physiological analysis of antennular chemoreception. Previous electrophysiological studies indicate antennular sensitivity to organic substances of low molecular weight in this species (Laverack, 1964; Levandowsky and Hodgson, 1965), and in the

American lobster, *Homarus americanus* (Ache, 1972; Shepheard, 1974). These substances at least partially elicit feeding behavior in lobsters of both species (personal observation; Mackie, 1973; McLeese, 1973). Behavioral studies of Carr and Gurin (1975) indicate that the chemical nature of major feeding stimulus for the shrimp, *Palaemonetes pugio,* varied with the type of stimulatory extract employed. The latter necessitates a survey of a series of stimulus types to fully understand potential macromolecular sensitivity of antennular chemoreceptors.

The present study surveys the sensitivity of antennular chemoreceptors of *Panulirus argus* to extracts and body fluids of potential food organisms both before and after fractionation by ultrafiltration. Data presented indicate that receptors sensitive to these extracts occur on the lateral antennular filaments and that, for all extracts and fluids tested, components of less than *ca.* 10,000 molecular weight are significantly more stimulatory than the components of higher molecular weight. Further, the stimulatory levels of the lower molecular weight components do not differ significantly from those of the full extracts or fluids.

Materials and Methods

Animal maintenance

Locally caught specimens of *Panulirus argus* were maintained in 150 gal. tanks of recirculating artificial sea water and fed frozen shrimp every third day.

Recording procedure

The preparation was the aesthetasc-containing distal 5–6 cm section excised from the lateral antennular filaments of adult lobsters. The filament was clamped through a silicon septum in a lucite recording chamber with the aesthetasc-containing annuli projecting into a tubular compartment carrying a continuous flow (10 ml/min) of reagent-grade artificial sea water into which various potential stimulants were introduced. The septum also isolated the stimulus-containing flow from a second compartment containing about 10 ml saline into which the filament's proximal end projected. Oxygen-saturated *Panulirus* saline (Mulloney and Selverston, 1974) perfused the filament *via* intralumenal cannulation. Perfusion extended viability of the otherwise short-lived preparation up to 3 hr post excision. Removal of the 3–4 most proximal cuticular annuli exposed the antennular nerve for subsequent teasing of fine nerve bundles onto platinum monopolar hook-type recording electrodes. Lifting into air provided sufficient resistance for recording 30–70 μV potentials from active chemosensory fibers. A Ag-AgCl pellet grounded the 10 ml bath. Neural activity was displayed on conventional recording equipment and stored on magnetic tape for subsequent photography and analysis.

Preparation and ultrafiltration of stimulants

The following live marine animals were used for preparing the indicated extracts or body fluids:

Mollusca
- Coquina (*Donax variabilis*) ..... entire crushed animals
- Oyster (*Crassostrea virginica*) ..... soft parts
  mantle fluid

Arthropoda
Blue crab (*Callinectes sapidus*) ..... claw muscle
hemolymph serum
Pink shrimp (*Penaeus dourarum*) ..... abdominal muscle

Echinodermata
Sea urchin (*Arbacia punctulata*) ..... soft parts

Chordata
Striped mullet (*Mugil cephalus*) ..... muscle

Extracts (3:1, volume:weight) were prepared by cutting up tissue in cold 1% NaCl and shaking for 30 minutes in an ice bath followed by centrifugation at 4° C and filtration of supernatants through Whatman #1 paper. A 100:1 extract of Tetramin (Tetra Sales Corp., Hayward, California-dry flaked fish food containing both animal and plant material) was prepared by adding flakes to 1% NaCl and treating as above. Mantle fluid of oyster and hemolymph serum of crab were prepared as described by Carr *et al.* (1974).

Each extract or body fluid was ultrafiltered at 4° C with 30 psi of N_2 through an Amicon UM-10 membrane retaining molecules greater than *ca.* 10,000 molecular weight. Each ultrafiltration provided two fractions as follows: a retentate containing substances of greater than *ca.* 10,000 mol wt and an ultrafiltrate containing smaller molecules. Each of these fractions and remainder of each total extract or fluid was divided into 5 ml aliquots and frozen until used. Single aliquots were thawed just prior to use and serially diluted with reagent-grade artificial sea water to provide a concentration series of that stimulant type. All stimuli were brought to ambient temperature (23° C), the temperature of the carrier flow, immediately before use since preliminary studies indicated a marked temperature sensitivity of antennular chemoreceptors.

Experimental protocol

Brief applications of a particular extract or fluid (and a seawater control) identified active chemosensory units as bundle searching proceeded. Bundles containing active units were then further subdivided until discrete unit activity could be resolved over background noise. An effective, yet nonsaturating concentration of test stimulus for a particular bundle was selected and 50 μl volumes each of the total extract, the retentate fraction, the filtrate fraction, and a repeated total extract were then applied to the preparation at the test concentration. A 2 min wash period separated each 50 μl application. Failure to obtain a terminal response to full extract equal in intensity to the initial full extract response voided the data of a series. Individual antennular preparations were tested with a single extract. The data presented on a given type of extract or fluid represent units from at least five different antennular preparations. To test for the possible loss of components labile to the ultrafiltration process, selected antennular preparations were used to compare the activity of each total extract or fluid with that of a 1:1 mixture of the retentate and ultrafiltrate.

TABLE I

Comparison of antennular chemosensitivity in P. argus *to extracts or fluids of potential food organisms before and after ultrafiltration into* >ca. *10,000 (retentate) and* <*ca. 10,000 (filtrate) molecular weight fractions.*

Extract type	Number of tests	$\bar{X}$ activity ratios		F*	*t*-test scores	
		Retentate: total	Filtrate: total		Retentate *vs* filtrate*	Filtrate *vs* total extract**
Coquina	8	0.37	0.98	14.43	6.62	0.21
Oyster-Fluid	9	0.28	0.95	12.81	5.70	0.40
Oyster-Tissue	14	0.22	0.84	11.10	6.45	1.28
Crab-Muscle	9	0.37	0.86	11.27	6.43	1.42
Crab-Serum	9	0.28	1.04	8.74	5.00	0.29
Shrimp	11	0.20	0.97	17.53	7.39	0.34
Urchin	8	0.12	0.96	17.09	58.56	0.34
Mullet	10	0.29	0.92	18.09	7.76	0.86
Tetramin***	7	0.15	1.00	11.21	4.17	0.00

* All values significant at the 0.01 level (single-sided).
** All values not significant, with $P \geqq 0.10$ level (single-sided).
*** A commercially-prepared fish food, containing both animal and plant material.

Data analysis

Lacking evidence as to what parameter(s) of afferent spike trains encode(s) chemosensory information in the lobster, we used the total spike count as the least variable and, tentatively, the most descriptive measure of responsiveness. The "response" of a nerve bundle therefore represents the total number of spikes elicited in that bundle on application of a given stimulus. Variability in the response of nerve bundles to a particular extract and to its ultrafiltration fractions was determined with a single classification analysis of variance and, if significant, specific treatments were compared further by a *t*-test. Analyses were performed on raw data. Tabulated values represent "mean activity ratios" in which the responses of a bundle to filtrate and to retentate are normalized to that bundle's mean extract response (the average response to initial and terminal total extract applications) prior to averaging.

RESULTS

Data are reported from 85 nerve bundles, each containing from one to approximately 12 active chemosensory units. Table I summarizes the overall results. For each of the nine types of extracts and fluids, the mean response to filtrate fractions was significantly greater than to retentate fractions. Comparisons of the response magnitudes for filtrates and retentates *vs* total extracts or fluids show ratios ranging from 0.84 to 1.04 for filtrates and only 0.12 to 0.37 for retentates. Further, for each type of extract or fluid, the mean response to filtrate fractions did not differ significantly from that elicited by the total extract. Thus, while both high and low molecular weight components are stimulatory, the major stimulants are present in the lower molecular weight component.

The greater stimulatory capacity of the lower molecular weight fractions did not result from the ultrafiltration procedure itself. In control trials, combined retentate and filtrate fractions elicited responses not significantly different from those elicited by the total extract or fluid. In 3 to 7 selected nerve bundles tested for each type of extract or fluid, 1 : 1 mixtures of the two fractions elicited responses with magnitudes 0.83 to 1.28 times that of the respective total extract responses.

Analysis of single unit activity provides further insight into the nature of the observed responses. Twenty-four single units were selected from the data presented in Table I and further analyzed. These include responses of bundles containing only single active chemoreceptors, and single active chemoreceptors selected from multi-fiber bundles containing sufficiently few units to allow discrete unit resolution (see Figure 1). Chemosensitive units were consistently small diameter fibers judging from their 100 μV-range spike amplitudes recorded with hook electrodes. Table II summarizes the single unit responses. The data were first analyzed to describe the basic response of actual chemoreceptors to total extract stimulation. Individual units responded consistently although interunit variability was appreciable. In general, units increased their rates of firing on total extract stimulation from spontaneous levels of 0–2 impulses/sec to maximum levels of 8–82 impulses/sec, subsequently decaying back to pre-stimulation levels over 0.7–12 seconds (Fig. 1, top 2 traces). Response duration and maximum spike frequency increased with stimulus concentration from thresholds of 10^{-4} to 10^{-5} times stock

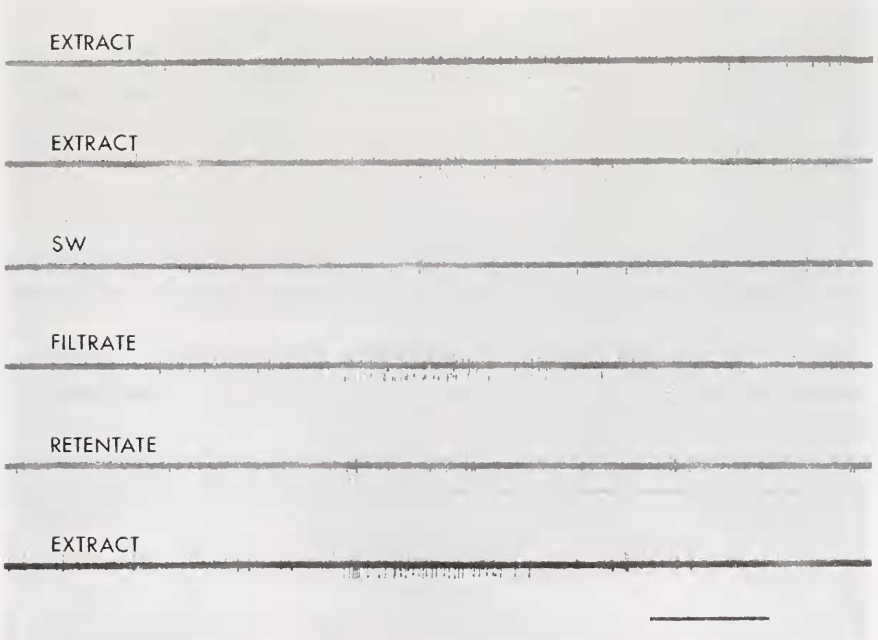

FIGURE 1. Extracellular records of chemosensory activity in *P. argus* lateral antennular filament in response to Tetramin (see text for composition) extract stimulation, 10^{-2} times stock concentration. Time calibration is 1.0 sec.

TABLE II

Single unit responses of antennular chemoreceptors in P. argus *to extracts or fluids of potential food organisms before and after ultrafiltration into* >ca. *10,000 (retentate) and* <ca. *10,000 (filtrate) molecular weight fractions.*

Unit number	Extract type	Number of spikes			Activity ratios	
		$\bar{X}$ total	Retentate	Filtrate	Filtrate: $\bar{X}$ total	Retentate: $\bar{X}$ total
1	Coquina	42	10	44	1.05	0.24
2	Oyster-tissue	108	7	79	0.73	0.06
3	Oyster tissue	15	6	12	0.80	0.40
4	Oyster tissue	20	7	14	0.70	0.40
5	Oyster tissue	9	3	3	0.33	0.33
6	Oyster tissue	33	0	30	0.91	0.00
7	Oyster-fluid	13	1	12	0.92	0.08
8	Oyster-fluid	34	14	50	1.47	0.41
9	Oyster fluid	68	0	29	0.43	0.00
10	Crab-muscle	9	14	8	0.89	1.56
11	Crab-muscle	38	9	23	0.61	0.24
12	Crab-serum	9	2	3	0.33	0.22
13	Crab-serum	116	6	99	0.85	0.05
14	Shrimp	170	14	144	0.84	0.08
15	Shrimp	16	5	17	1.06	0.31
16	Shrimp	122	8	82	0.67	0.07
17	Shrimp	84	6	92	1.10	0.07
18	Urchin	28	11	29	1.04	0.39
19	Urchin	16	1	18	1.13	0.06
20	Mullet	23	0	20	0.87	0.00
21	Mullet	26	25	24	0.92	0.96
22	Tetramin	28	0	28	1.00	0.00
23	Tetramin	33	3	30	0.91	0.11
24	Tetramin	35	1	32	0.91	0.03

extract concentrations. Response latencies obtained in our apparatus varied from 2.0 to 12.4 sec post-stimulus introduction. (The minimum delay for the stimulus front to traverse the entire length of the excised filament was 1 sec as determined visually by dye flow.).

Single units were then analyzed for differences in sensitivity to the two fractions. Twenty units responded more to filtrate than to retentate stimulation, one responded more to retentate stimulation, and three showed essentially no difference in response. Filtrate: total activity ratios among units more sensitive to filtrate stimulation varied from 0.43–1.47. No evidence of temporal patterning differing from that described for the total extracts was apparent in either the filtrate or the retentate responses.

DISCUSSION

Comparisons of the stimulatory capacity of high and low molecular weight fractions from 9 extracts and body fluids show clearly that in each case the major stimulants for antennular chemoreceptors of *Panulirus argus* are substances of less than *ca.* 10,000 mol wt. If, as considered below, the antennules are primarily distance

chemoreceptors, it seems unlikely that macromolecules play a major role in the attraction of *P. argus* to distant food sources. Unfortunately, behavioral correlates of the present study on *P. argus* are not available. Behavioral studies of food-finding in two other decapods, *Homarus gammarus* and *Carcinus maenas,* demonstrate that synthetic mixtures of low mol wt components present in food organisms provide attractants as effective as aqueous extracts of the organisms (Mackie, 1973; Shelton and Mackie, 1971). In both of these studes, however, macromolecular components were not systematically eliminated as potential attractants. Carr and Gurin (1975) concluded that the attraction of the natantian, *Palaemonetes pugio,* to human serum was due to the combined effects of both macromolecules (proteins) and substances of less than 1000 mol wt. A mixture of the 37 major low mol wt components of human serum was only about one-eighth as effective as the total serum itself. Further analysis of chemosensitivity in *Palaemonetes* using extracts similar to those employed in the present study showed that whereas the major stimulants in three extract types were components of less than 1000 mol wt, those in two other extract types were components of greater than 1000 mol wt. Our current data with *P. argus* support the hypothesis that the chemical signals used for food-finding in this species are not macromolecules, though they may be small proteins or peptides.

Bardach (1975) recently reviewed and listed the known and suspected chemical signals of marine organisms. Molecules of less than 1000 mol wt dominate the list. As noted by Carr *et al.* (1974) most studies, both behavioral and physiological, focus specifically on such small molecules as potential stimulants without systematically eliminating components of higher mol wt by "working down" from a complete stimulant such as an aqueous extract or a body fluid. Several investigators have fractionated aqueous extracts or body fluids that stimulate feeding behavior and have shown that, in certain cases, macromolecules make important contributions to their activity. Gurin and Carr (1971) studied the chemical stimulation of feeding behavior in the gastropod, *Nassarius obsoletus,* and found that the major stimulant in oyster mantle fluid was a glycoprotein of *ca.* 100,000 mol wt that was effective at concentrations as low as 10^{-10} M. Magnum and Cox (1971) reported a glycoprotein of about 20,000 mol wt that contributed significantly to the feeding response of the tube-dwelling polychaete, *Diopatra cuprea.* Heeb (1973) found protein fractions of $>$ 100,000 mol wt from two mollusc extracts to be major stimulants of the "humping reflex" and food-searching behavior in the seastar, *Asterias forbesii.* Alarm responses in the aquatic gastropod, *Helisoma duryi,* are apparently elicited by conspecific tissue components of *ca.* 100,000 mol wt (Snyder, 1967, cited in Bardach, 1975). Since at least some of these macromolecular stimuli elicit oriented locomotion or exhibit extremely low effective concentrations (10^{-10} M in *Nassarius*), the difference between the findings cited above and those of the present study cannot be explained on the basis of distance chemoreception *vs* contact or close-range chemoreception. Regarding prior demonstrations of the stimulatory capacity of macromolecules, Mackie (1975) proposed the interesting idea that the slow fade out time of large molecules, resulting from their low diffusion constants, may make them more favorable as chemical signals in slow-moving organisms such as gastropods and seastars than in faster-moving organisms such as decapod crustaceans.

It is generally assumed that crustacean antennules function in distance chemoreception, *i.e.,* in the detection of low concentrations of water-borne odorants which alert and/or initiate oriented behavior in the recipient organism (for review see Hazlett, 1971). Exceptions apparently exist, as antennules are reported not to mediate food location in a freshwater crayfish (Ameyaw-Akumfi and Hazlett, 1975). That lateral antennular filaments effect distance chemoreception in *P. argus* is supported by ablation studies (unpublished data) in which lobsters lacking this appendage fail to locate a source of shrimp extract in an olfactometer designed specifically to assay the chemotaxic components of feeding behavior. Similar results are reported for the American lobster (McLeese, 1973). As noted elsewhere (Ache, 1975), ablation with subsequent loss of response is not a definitive technique as the presumed chemoreceptor could be mediating nonchemical signals required concomitantly with chemosensory input *via* other receptor structures for orientation. This alternative has not been ruled out in *P. argus* but knowledge that chemoreceptors with thresholds as low as 10^{-11} M glycine and L-glutamic acid occur on the lateral filament of *P. argus* (Price and Ache, in preparation), it remains a less probable explanation of the ablation results.

Previous attempts at recording chemosensory activity from crustacean antennules have been limited by preparation viability. Our results, obtained from perfused preparations, extend and support those of previous studies that antennular (lateral filament) chemoreceptors of *P. argus* (Laverack, 1964; Levandowsky and Hodgson, 1965), the American lobster (Ache, 1972, Shepheard, 1974) and the brachyuran, *Plagusia dentipes,* (Ai and Takei, 1973) are small diameter fibers yielding fast, 30–100 μV extracellular spikes. Small spikes coupled with long response latencies and concentration-dependent decay periods characterize chemosensory responses from those of the ever-present mechanosensory fibers that occur in the same fiber bundles.

No attempt was made to compare response spectra of the individual fibers to the various extract types, but unit responses can be compared relative to the retentate and ultrafiltrate fractions used in the present study. While all but one of the single units responded much more strongly to the filtrate fractions, most of the units also showed some weak response to the retentate fractions. It is likely that part of the weak response obtained with retentates was due to the presence of small concentrations of low molecular weight substances not removed by the ultrafiltration process. Another explanation for the activity of the retentates is that proteins and other large molecules in these fractions possess functional groups which bind with receptor sites for low mol wt compounds. However, the overall contribution of the large molecules to the effectiveness of the total extracts and fluids was minimal as indicated by the fact that each ultrafiltrate, devoid of molecules of greater than *ca.* 10,000 mol wt, was virtually as stimulatory as the unfractionated extract or fluid. Further analyses are in progress concerning the specific nature of the substances in shrimp ultrafiltrate that stimulate antennular chemoreceptors in *P. argus* (Johnson and Ache, in preparation). These analyses indicate that the major stimulants have molecular weights of less than 1000 and are amino acid-like in nature.

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Summary

Antennular chemoreceptors in the spiny lobster, *P. argus,* were surveyed electrophysiologically for responsiveness to natural stimuli of different molecular weights to gain further insight into the stimulatory role of macromolecules. Extracts and body fluids from eight potential food organisms were prepared and tested both before and after fractionation by ultrafiltration. Data presented verify chemosensitivity of lateral antennular filaments and show that for all extract types. the components of low molecular weight ($< ca.$ 10,000) were significantly more stimulatory than the components of higher molecular weight; and stimulus values of low molecular weight fractions did not differ significantly from those of the unfractionated extracts.

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